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Emergence of porcine epidemic diarrhea viruses with the novel S genes in Tibetan pigs in the Qinghai-Tibetan plateau in China

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ABSTRACT

The purpose of present study was to investigate the prevalence and genetic variation of porcine epidemic diarrhea virus (PEDV) in Tibetan pigs on the Qinghai-Tibetan Plateau in 2018. The PCR yielded a significantly high detection rate (38.34%, 95%CI = 31.5–45.6%) for PEDV from 193 fecal samples from Tibetan pigs. The novel PEDVs were discovered in Tibetan pigs and seven complete S genes were obtained and analyzed. The unique multiple mutations were detected in S genes of PEDV from Tibetan pigs, one of which led to a new amino acid substitution of a neutralizing epitope. Phylogenetic analysis showed that seven S genes had significant genetic distance to other PEDV. Specially, two S genes formed a novel subgroup on the genogroup 2 (G2) branch, of which same recombination event occurred between different strains from genotype G2. The remaining five S genes formed a new subgroup on the G1 branch, among which distinct recombination events occurred between genotypes G1 and G2 strains. The result indicated that the new recombination events were detected in the S genes of PEDV from Tibetan pigs, which could be circulating in the Qinghai-Tibetan Plateau. Notably, the four complete PEDV genomes obtained in this study had an identical recombination region spanning S2, ORF3 and E genes. This is the first report of the crossover regional recombination event in PEDV genome. Our findings not only augmented current understanding of the genetic evolution of PEDV, but also indicated that new variants of PEDV strains have been emerging in Tibetan pigs.

1. Introduction

Porcine epidemic diarrhea virus (PEDV) is a *Coronaviridae* family member (genus *Alphacoronavirus*) first identified in England in 1971 and followed by outbreak of the disease in many countries, resulting in large economic losses (Pensaert and de Bouck, 1978; Sato et al., 2011; Wen et al., 2018). This virus causes porcine epidemic diarrhea, an acute and highly contagious enteric disease characterized by watery diarrhea, vomiting and high mortality in piglets (Diep et al., 2018; Li et al., 2016). Since 2010, severe PEDV outbreaks caused by highly virulent strains have spread rapidly nationwide (Lee et al., 2010; Li et al., 2012). Variant PEDV strains have also been reported in various countries, including the USA, Germany, and South Korea (Hanke et al., 2015; Lee and Lee, 2014; Stevenson et al., 2013). Consequently, PEDV has received increasing attention since the emergence of novel PEDV variants in many swine-breeding countries worldwide.

PEDV is an enveloped, single-stranded, positive-sense RNA virus of approximately 28 kb in size, which contains seven open reading frames (ORFs) in the following order: ORF1a, ORF1b, the spike (S) glycoprotein, ORF3 (a hypothetical protein gene), envelope (E), membrane (M) and nucleocapsid (N) (Crawford et al., 2015; Yang et al., 2014). The S protein of PEDV, which is a membrane glycoprotein, stimulates the production of neutralizing antibodies by the host, and its dramatic mutational changes regularly cause antigenic and pathogenic changes in new PEDV variants (Park et al., 2014; Yu et al., 2018). The S protein can be divided into two main amino acid (aa) subunits: S1 (aa positions 1–789) and S2 (aa positions 790–1383) (Cao et al., 2015; Sun et al., 2015). The S1 functional region contains two domains, the N-terminal domain (S1-NTD; 21–324 aa) and the C-terminal domain (S1-CTD; 253–638 aa), both of which can potentially function as receptor-binding domains (Deng et al., 2016; Li, 2015; Su et al., 2016). Thus, the S1 is more genetically diverse than S2 and is utilized to determine the

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genetic relatedness of different PEDV viruses (Chen et al., 2014a; Sun et al., 2015). Based on the different evolutionary analyses of the S gene, PEDV is mostly classified as genogroup 1 (G1), which contains classic S-INDEL strains and genogroup 2 (G2) with pandemic non S-INDEL strains, and is further divided into four subgroups; namely, G1a, G1b, G2a and G2b (Wang et al., 2016b).

Recombination commonly occurs in PEDV strains (Boniotti et al., 2016; Jarvis et al., 2016; Lin et al., 2016). The breakpoint recombination events frequently occur in PEDV genome in the following six main regions: 5′ UTR, ORF1a, ORF1b, S, N and 3′ UTR (Lin et al., 2016). With the increasing of emergence of PEDV variants, various types of recombination events have also been detected in PEDV strains (Chiou et al., 2017; Lin et al., 2016; Wang et al., 2016a). Indeed, studies have shown that recombination among the different strains is considered a major evolutionary pattern that allows the emergence of new strains with altered virulence and immunogenicity profiles in PEDV (Chen et al., 2017; Jarvis et al., 2016).

Tibetan pigs mainly live on the Qinghai-Tibet Plateau with an altitude of 3,500-4,500 m in China, and have the characteristics of coping with low-temperature and inferior food (Li et al., 2017). Tibetan pigs are the only high-altitude pasture pig breed, making them the main source of economic income for Tibetans (Gao et al., 2018). Severe diarrhea is a common disease that reduces productivity and increases loss by death in Tibetan piglets (Dong et al., 2018). However, the current viral pathogens associated with diarrhea in Tibetan pigs remained unclear. In this study, we collected 193 fecal samples from Tibetan pigs from 13 different farms on the Qinghai-Tibet Plateau to investigate the epidemic situation of PEDV. Also, the S gene and complete PEDV genome were characterized to study the genetic and evolutionary relationships among the isolates.

2. Materials and methods

2.1. Sample collection and RNA extraction

In 2018, a total of 193 fecal samples were collected from 10 to 40-day-old Tibetan piglets on 13 different farms in four different regions in the Ganzi Tibetan autonomous prefecture of Sichuan province (Table S1), which were located in the southeastern margin of the Qinghai-Tibetan Plateau. The herd size was usually small-scale farm, and the Tibetan piglets were free-range and not vaccinated against PEDV, transmissible gastroenteritis virus (TGEV), porcine group A rotavirus (PoRV) and porcine deltacoronavirus (PDCoV). Of these fecal samples, 129 were collected from diarrheal piglets with severe watery diarrhea, sharking and dehydration, and 64 were collected from clinically healthy animals. After sampling, 10% (wt/vol) fecal suspensions were prepared using sterile phosphate-buffered saline (PBS). Supernatants were separated after sample centrifugation, and the samples obtained were stored at −80 °C until RNA extraction. Total RNA was isolated from the samples using the QIAamp Viral RNA Mini Kit (QIAGEN, Germany), following the manufacturer's instructions. Reverse transcription was performed using SuperScript III reverse transcriptase (RT) (Invitrogen, USA) and random hexamers (Invitrogen), following the manufacturer's protocol, and the cDNA was synthesized and stored at −20 °C.

2.2. Detection of PEDV in fecal samples from Tibetan pigs

The PCR primers (Table S2) were used to determine the positivity rate for PEDV and three other diarrhea-associated viruses including TGEV, PoRV and PDCoV (Zhang et al., 2014). The PCR detection was performed in a 25 μL volume, which included 1 μL of template, 1 μL of each primer (10 pmol), 8 μL of ddH_2O, and 12.5 μL of Quick Taq HS DyeMix at 1× concentration (Toyobo, Japan). The PCR conditions were set at 94 °C for 2 min, 35 repeats of denaturing at 94 °C for 30 s, annealing at 51 °C for 30 s, extension at 68 °C for 1 min, and a final extension at 72 °C for 8 min. The RT-PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized by ultraviolet illumination. Finally, the PCR products consistent with the expected size were sent to Sangon Biotech (Shanghai, China) for sequencing.

2.3. Cloning, sequencing and genome assembly of PEDV from Tibetan pigs

Five primers (Table S2) were designed to amplify the entire S gene, based on the conserved regions determined by multiple sequence alignment analysis of the reference strains from GenBank. An additional 22 pairs of primers (Table S2) were designed to amplify the complete genome from the PEDV-positive products. Briefly, the PCR amplified target fragments that were purified using a gel extraction kit (OMEGA, USA) were ligated to a pMD19-T vector (TaKaRa, Dalian, China). The resultant plasmids were transformed into *Escherichia coli* DH5α cells (TIANGEN, China) and sequenced (Sangon Biotech). SeqMan (Lasergene, USA) was used to assemble and analyze the sequencing data.

2.4. Molecular characteristics and phylogenetic analysis of PEDV from Tibetan pigs

To perform a comparative analysis of the genomic organization of PEDV, putative ORFs were predicted using the ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The homologies of the nucleotide and deduced amino acid sequences were analyzed using MegAlign (Lasergene). The CLC Sequence Viewer 6.0 software was used to align the nucleotide and deduced amino acid sequences of the complete S gene. Phylogenetic trees were constructed using the neighbor-joining method with 1,000 replicates and bootstrap values in MEGA 7.0.

2.5. Recombination analysis

Recombination detection program (RDP 4.0) with seven recombination detection methods (RDP, GENECONV, BOOTSCAN, MaxChi, Chimaera, SISCAN and 3Seq) was used to predict recombinant events between full-length S gene and complete genome from Tibetan pigs PEDV strains and other reported strains (Martin et al., 2015). Only those recombination events supported by more than five programs were considered to avoid dependence on a single methodology. The window size was set to 20 bp, the highest acceptable *P* value was 0.05, and the detection of the recombination events were applied between sequences sharing 0% and 100% identities. The detected recombination events were further evaluated by SimPlot 3.5.1 software. Finally, phylogenetic trees were constructed to further determine the evolutionary relationships and potential recombination events between Tibetan pig PEDV strains and their putative parental strains.

3. Results

3.1. PEDV detection in fecal samples from Tibetan pigs

In total, 193 fecal samples from Tibetan piglets were collected from 13 different farms for PEDV detection. We also tested for three other diarrhea-associated viruses in the samples; namely TGEV, PoRV and PDCoV. By RT-PCR, PEDV was found in 38.34% (74/193, 95% confidence interval, CI = 31.5–45.6%) of all samples. The PEDV positivity rate for the 129 diarrheal samples was 42.64% (55/129, 95% CI = 34.0–51.6%), while that of clinically healthy animals was 29.69% (19/64, 95% CI = 18.9–42.4%). PoRV was found in 55.96% (108/193, 95% CI = 48.7–63.1%) of the samples, and none were positive for TGEV or PDCoV (95% CI = 0–3.4%). Additionally, the PEDV-PoRV co-infection rate was 35.25% (68/193, 95% CI = 28.5–42.4%) in the diarrheal samples. PEDV positive samples were detected in all 13 of the different farms. These results indicated that PEDV was one of the main

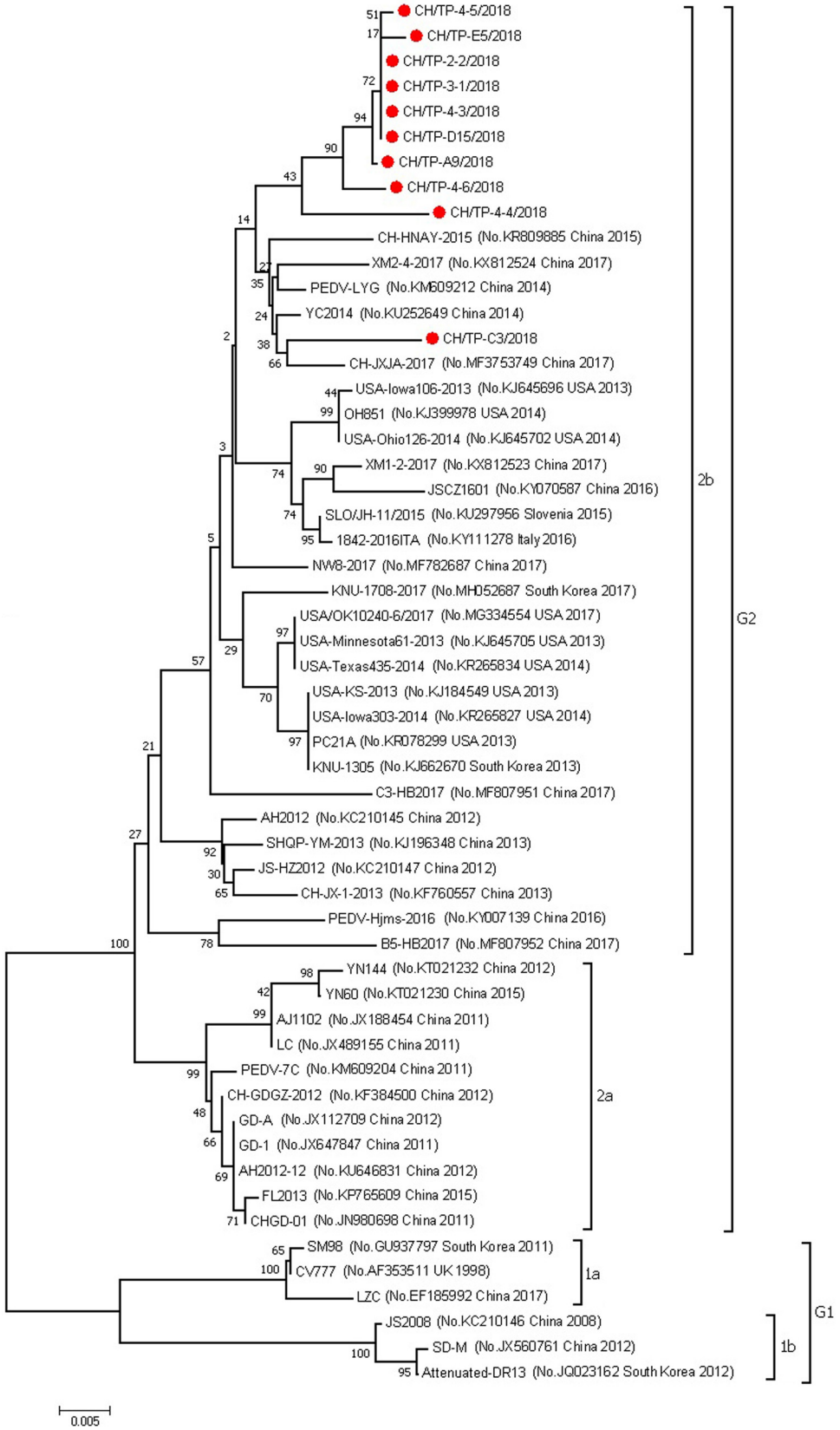

Fig. 1. Phylogenetic tree based on sequence alignment of partial S1 gene sequences (positions 788–1654 bp) from the Tibetan pigs PEDV. The tree was constructed by the Neighbor-joining method and by 1,000 replicates with the bootstrap values in MEGA 7.0 program. Red circles indicate the 10 S1 gene sequences from the Tibetan pigs. The reference sequences obtained from GenBank are indicated by strain abbreviations, and the bracket contains the strain information, including GenBank accession number, country and collection year.

causative agents of diarrhea in the Tibetan pigs.

3.2. Phylogenetic analysis of partial S1 genes of PEDV in Tibetan pigs

As a key virulence factor, the S gene was frequently utilized as a valuable molecular marker for determining genetic relatedness among PEDV strains, for investigating epidemiology situations, and for vaccine development (Lawrence et al., 2014; Lee and Lee, 2017). To study the evolution and genetic diversity of PEDV from Tibetan pigs, we firstly used a pair of primers designed for the S1 partial gene (positions 788–1654 bp) to detect PEDV from 13 different farms in four regions. The result exhibited that only 10 S1 genes (873 bp) were successfully obtained from four different farms. According to the homology analysis, the 10 partial S1 genes sequences shared 95.9% to 100% nucleotide sequence identities with each other, and 92.0% to 98.4% nucleotide sequence identities with the reported sequences. A phylogenetic tree was constructed based on partial nucleotide sequences of S1 genes (Fig. 1). The results exhibited that the 10 PEDV strains from the Tibetan pigs fell into G2b, but nine strains of them were clustered into a new cluster, the remaining one strain clustered with Chinese pandemic strain, which suggested that the nine PEDV strains with novel S genes possibly existed in Tibetan pigs.

3.3. Molecular characteristics of the seven S genes of PEDV

To understand the genetic characteristics of S genes in this study, the nine novel S1 genes were selected to amplify the entire S gene. The result showed that seven full-length S genes (CH/TP-2-2/2018, CH/TP-3-1/2018, CH/TP-4-3/2018, CH/TP-4-4/2018, CH/TP-A9/2018, CH/TP-D15/2018 and CH/TP-E5/2018) were successfully PCR amplified from four farms and deposited in the GenBank database under the accession numbers MH593890-MH593896. The S genes were 4,146-4,161 nucleotides in length, and shared 96.1% to 99.9% nucleotide sequence identities and 93.8% to 99.9% amino acid identities with each other, and 93.1% to 98.3% nucleotide sequence identities and 89.9% to 98.4% amino acid identities with other 78 S gene sequences.

To further characterize generic variation in the seven S genes, the sequence alignment of the deduced amino acids of the S proteins were compared with those for some representative reference PEDV strains. The sequence alignment showed that the seven S genes possessed several obvious mutations (482V→N, 772 P/R→L/S, 1004L→M) (Fig. 2a). With the exception of CH/TP-4-4/2018 strain, two amino acid mutations (347L→F, 420L→I) (Fig. 2b) were detected in six of the PEDV S genes. Excepting for CH/TP-4-4/2018 and CH/TP-D15/2018 strains, the remaining five strains were found to contain additional six significant amino acid mutations (141-143TVN/TAN→SSG, 148T→S, 185 G/H→K/R, 201R/K→I) (Fig. 2b). The above data exhibited that 11 unique amino acid mutations were identified in N-terminal domain (NTD) and C-terminal domain (CTD) regions among the seven S1 genes. On the other hand, the S protein contains the following four neutralizing epitopes: the CO-26 K equivalent (COE) domain (aa positions 499–638), the SS2 epitope (aa positions 748–755), SS6 (aa positions 764–771), and 2C10 (aa positions 1,368-1,374) (Chiou et al., 2017). In this study, two unique amino acid substitutions were found in the COE neutralizing epitope of the S gene from the seven PEDVs (Fig. 2a). Only one amino acid substitution (530 H/I→P) was identified in the newly emergent variant JSCZ1601 strain in China (Fan et al., 2017), with the other (535L→I) being first identified in the present study. Further comparative sequence analysis found that deletions and insertions (INDELS) in the amino acid sequences of CH/TP-4-4/2018 and CH/TP-D15/2018 strains were approximately the same as those of the strains located in the G2 cluster represented by the AH2012 strain (Fig. 2b). The amino acid INDELS in the remaining five strains were similar to those of strains belonging to the G1 cluster represented by the CV777 strain (Fig. 2b). These molecular characteristics of the seven S genes suggested that two different subgroups, G1 and G2, possibly existed in PEDV from the Tibetan pigs.

3.4. Phylogenetic and recombination analysis of the seven S genes of PEDV

Here, we constructed a phylogenetic tree for the full-length S gene using the seven PEDVs from the Tibetan piglets and the other reported 78 PEDV sequences. As shown in Fig. 3, the seven S genes of PEDV from the Tibetan piglets were divided into two subgroups between G1 and G2 branches and had obvious differences to those reported previously for PEDV. Both CH/TP-4-4/2018 and CH/TP-D15/2018 strains formed a single subgroup in the G2 cluster, and had a relatively distant genetic relationship with other strains located on G2. The remaining five PEDV strains fell into a single subgroup in the G1 cluster that were genetically diverse. The above results were in agreement with those of the molecular characterization, strongly suggesting that two distinct novel PEDV subtypes were circulating in Tibetan pig herds. Interestingly, CH/TP-4-3/2018 and CH/TP-4-4/2018 strains were from the same Tibetan pig farm, as were CH/TP-A9/2018, CH/TP-D15/2018 and CH/TP-E5/2018 strains, indicating that at least two distinct new emerging PEDV subtypes existed on the same farm. Therefore, we postulated that potential recombination events might exist in the PEDVs from the Tibetan piglets.

To identify potential recombination events in PEDV from the Tibetan pigs, two different types of potential recombination events were determined in seven S genes using RDP 4.0 and Simplot 3.5.1 software. Both strains CH/TP-4-4/2018 and CH/TP-D15/2018 located on the new branch of G2, had the same recombination event in the crossover region at nucleotide positions 3,357 and 3,780 of the C terminal region of S2 (Fig. S1). The putative parental strains of both strains were Chinese epidemic strains XM/2-4/2017 and LC, belonging to G2 cluster. Also, the remaining five strains located on the new branch of G1 possessed the same recombination event in a crossover site of nucleotide position 716 of the C terminal region of S1 (Fig. S2). The five strains had the same minor parent strain from G1 cluster, but the different major parental strains from G2 cluster, whose putative parental strains were from epidemic PEDV in China.

3.5. Molecular characteristics of the four complete PEDV genomes from Tibetan pigs

To obtain more precise information on the evolutionary relationship of the PEDVs from the Tibetan pigs, we successfully assembled four whole genomic sequences from the seven PEDVs with nucleotide lengths encompassing 28,022-28,041 bp (GenBank accession number: MK140811-MK140814). These viruses shared 98.7% to 98.9% nucleotide sequence identities and 98.2% to 98.6% amino acid sequence identities with other reported complete PEDV genome sequences. We were unable to obtain the remaining three complete PEDV genomic sequences, a finding possibly related to large genetic variation in the sequences. As can be seen in Table S3, we compared total nucleotide lengths and seven ORFs amino acid sequences of the four strains with classic CV777 and pandemic AH2012 strains. The comparative analysis showed that the total nucleotide lengths of four strains were different from that of two representative reference strains. The homology analysis showed that the four S genes from Tibetan pigs shared 93.6% to 95.1% amino acid sequence identities to CV777 strain, which was the lowest identities among seven ORFs of PEDV.

To understand the genetic evolution of the Tibetan pigs PEDV genome, we constructed phylogenetic trees based on the complete genome and the nucleotide sequences of six genes (i.e., ORF1a, ORF1b, ORF3, E, M and N) (Fig. 4). The results showed that the four PEDV strains from the Tibetan pigs fell into the G2b subgroup and clustered on a single new branch, as based on the whole genomic sequences (Fig.4a), which further indicated that the PEDV strains in this study were a novel strain. According to the ORF1a, E and M genes sequences (Fig. 4b, 4e and 4f), the four isolated strains all fell into the G2b subgroup. In contrast, the ORF1b, ORF3 and N genes phylogenetic trees

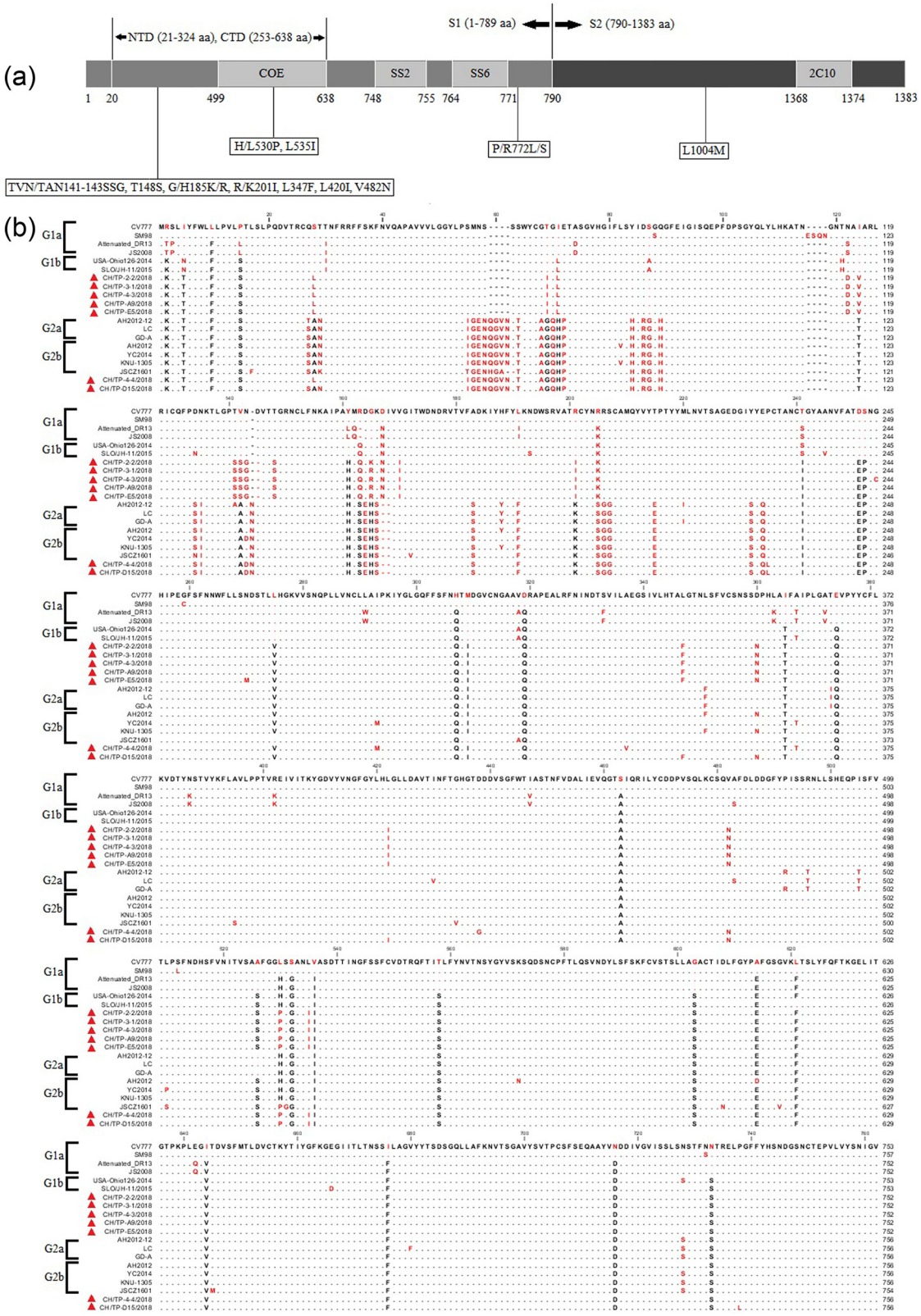

Fig. 2. Amino acid differences of the S protein in seven Tibetan pig PEDV strains and other reported PEDV strains. (a) Unique amino acid variations in the S gene from Tibetan pigs are framed in a box. The four neutralizing epitope regions in the S protein include the COE domain, and the SS2, SS6 and 2C10 regions. The S1 functional region contains two domains, the N-terminal domain (NTD; 21–324 aa) and the C-terminal domain (CTD; 253–638 aa). (b) Alignment of the deduced amino acid sequences of the S1 region (positions 1–789 aa) of seven complete S proteins in this study and representative PEDV strains from G1 and G2. Deletions and mutations are shown in red.

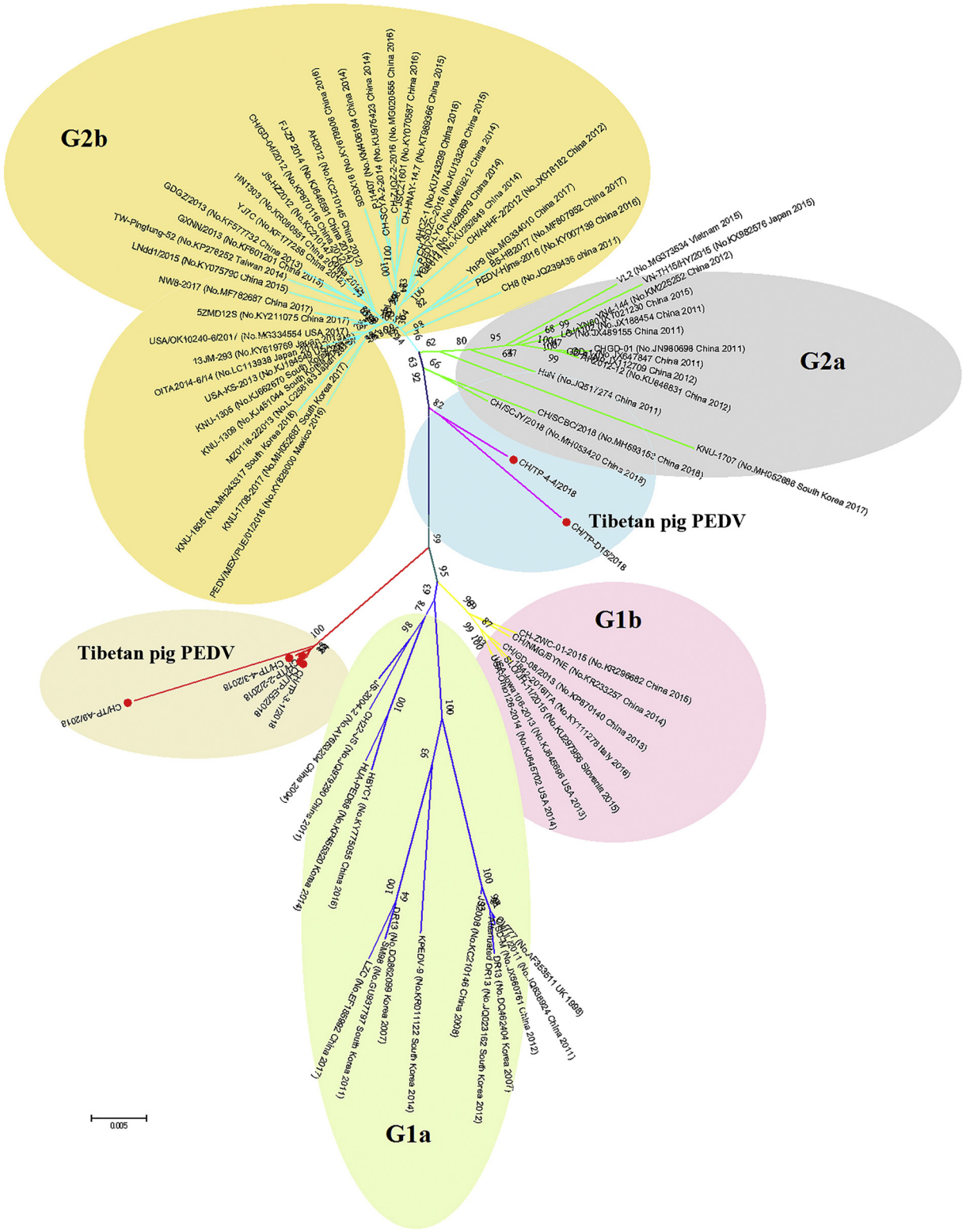

Fig. 3. Phylogenetic tree based on the nucleotide sequences of the entire S genes of seven PEDV strains isolated from Tibetan piglets and other 78 PEDV strains. The tree was constructed using the neighbor-joining method. The red circles indicates the S gene sequences of PEDV from the Tibetan pigs. The CH/TP-4-3/2018 and CH/TP-4-4/2018 strains were from the same farm. The CH/TP-A9/2018, CH/TP-D15/2018 and CH/TP-E5/2018 strains were from the same farm. The CH/TP-2-2/2018 and CH/TP-3-1/2018 strains were from the different farms. The reference sequences from GenBank are indicated by strain abbreviations, and the bracket contains the strain information, including GenBank accession number, country and collection year.

(Fig. 4c, 4d and 4 g) showed that the four PEDV strains fell into the G2a subgroup. The above results revealed that obvious differences in the phylogenetic relationships have occurred in the different PEDV genome regions, suggesting that the PEDV strains from the Tibetan pigs have undergone complex molecular genetic evolution.

The recombination analysis on the complete genome showed that potential recombinant events were detected in the four strains and a same crossover region at nucleotide positions 24,083 and 25,675 was

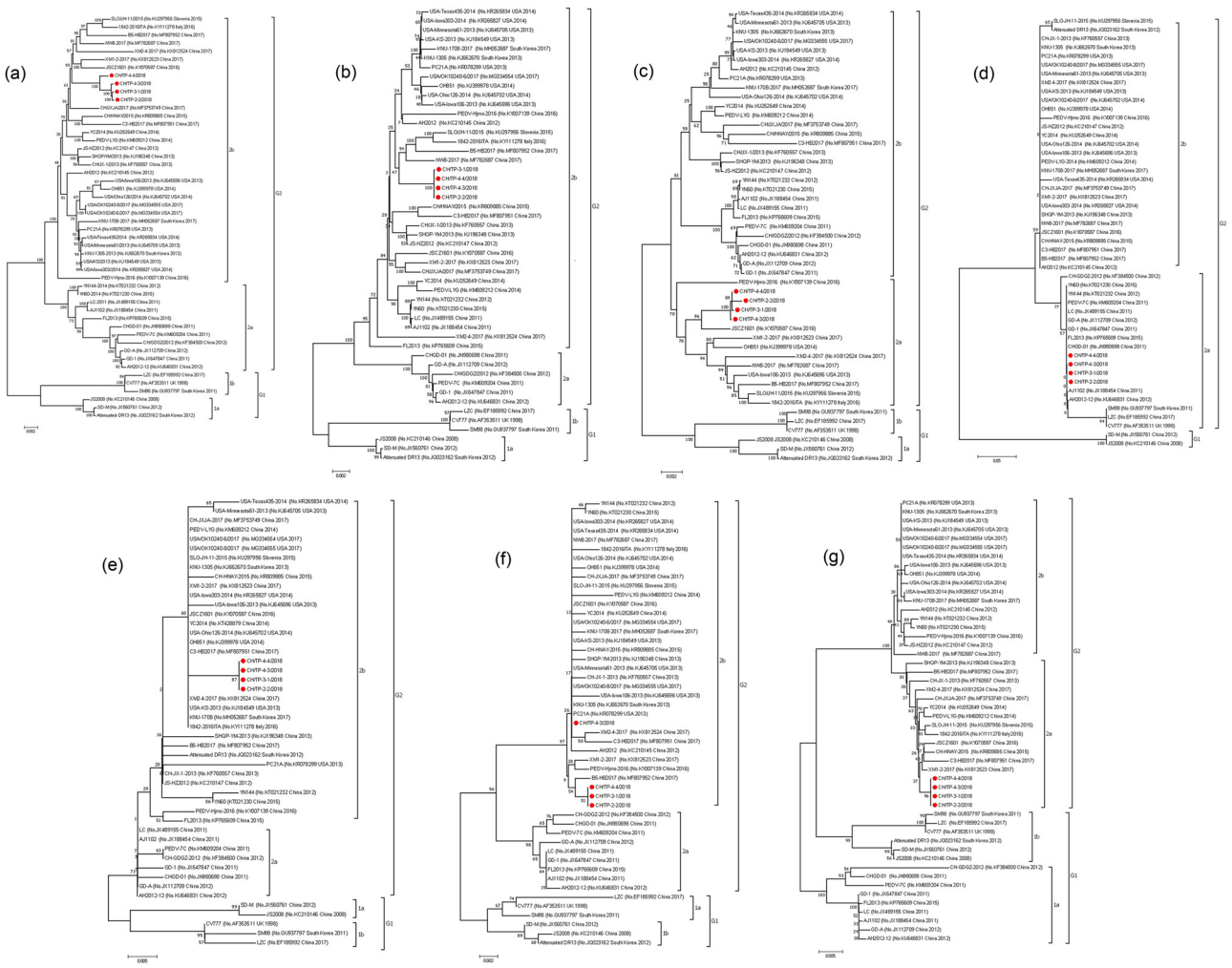

Fig. 4. Phylogenetic tree based on the nucleotide sequences of the complete genome (a) and the ORF1a (b), ORF1b (c), ORF3 (d), E (e), M (f), and N (g) genes from the PEDV strains isolated from Tibetan piglets and other reference strains. The tree was constructed using the neighbor-joining method. Red circles indicate the sequences from the viruses described in this study. The reference sequences obtained from GenBank are indicated by strain abbreviations, and the bracket contains the strain information, including GenBank accession number, country and collection year.

determined in them (Fig. 5a). The putative parental strains of the four PEDV strains were from G2a and G2b subtypes. Of note, the same crossover region contained a partial S2 gene, the complete ORF3 gene, and the near-complete E gene. The phylogenetic trees (Fig. 5b) based on the nucleotide sequences of the A and C regions of PEDV strains from the Tibetan pigs showed that the four recombinant strains clustered closely with major parent USA-Ohio126/2014, SLO/JH-11/2015 and KNU-1305 strains. Conspicuously, the phylogenetic tree for the B region showed discordant genetic phylogenetic relationships when compared with the other two trees. The CH/TP-2-2/2018, CH/TP-3-1/2018, CH/TP-4-3/2018 and CH/TP-4-4/2018 strains clustered with the minor parent AH2012-12 and CHGD-01 strains at the recombination region. Therefore, the significantly discrepant topologies of the phylogenetic trees supported the possibility of further recombinant events in these strains.

4. Discussion

The Tibetan pig is a special indigenous Chinese pig breed distributed in Sichuan, Qinghai, Gansu, and Tibet provinces (Wang et al., 2018). The cold and harsh environment might be a factor that accelerates microbial evolution and mutation in these animals (Yu et al., 2018). Recently, severe diarrhea in suckling piglets occurred in Tibetan pig farms in the highlands southwest of China. However, to date, only limited information on the viral pathogens associated with porcine diarrheal infections in Tibetan pigs could be available (Gao et al., 2018). In recent years, numerous studies have reported that variant PEDV strains have caused severe diarrhea and a massive death toll in piglets in China (Lee, 2015; Masuda et al., 2015; Yang et al., 2013a). This inspired us to investigate the epidemic situation of PEDV by testing the feces from Tibetan pigs. We found that the PEDV detection rate (38.34%, 95% CI = 31.5–45.6%) was clearly higher than that previously reported for Tibetan piglets (Wang et al., 2018). The above results indicated that the PEDV was emerging and causing porcine diarrhea in Tibetan pigs.

The S gene was frequently used as a molecular marker in studies on the genome characteristics of PEDV strains (Chen et al., 2013). Based on phylogenetic analysis of 10 partial S1 genes on four farms, we identified nine novel S genes in Tibetan pigs PEDVs in this study, suggesting that the novel PEDVs could be existed in Tibetan pigs. Following, the seven full-length novel S genes were amplified and analyzed, exhibiting the more complicated genetic variation than those of previously reported (Yu et al., 2018; Zuo et al., 2018). Comparative analysis of the amino acid sequences of the seven S genes from Tibetan pigs PEDVs revealed that most of the amino acid deletions and insertions were primarily located in the S1 domain, a finding consistent with previous reports on the S gene of highly pathogenic PEDV strains from domestic pigs (Fan et al., 2017; Lin et al., 2016). In particular, the unique amino acid

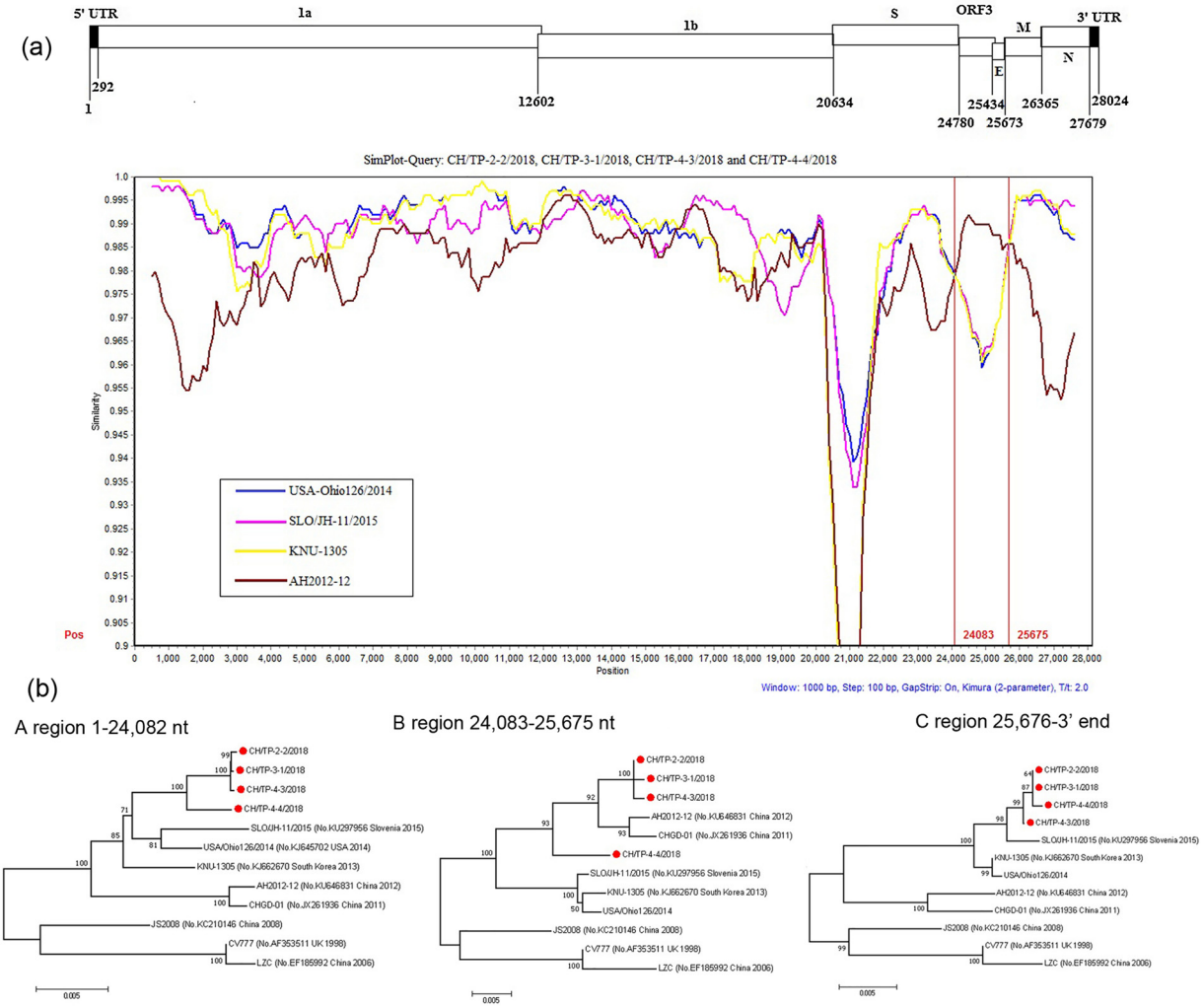

Fig. 5. Recombination analysis of the complete PEDV genomes from Tibetan pigs. (a). SimPlot analysis of the complete genome represented by CH/TP-2-2/2018 strain; window size, 200 bases; step, 20 bases. The crossover regions identified by SimPlot are consistent with the results from the RDP 4.0 analysis. The vertical axis indicates nucleotide sequence similarity (%) between the query strain and reference strains. (b) Phylogenetic trees for region A (nt 1 to 24,082), region B (nt 24,083 to 25,675) and region C (nt 25,675 to the end of the sequence) of the four Tibetan pig PEDV strains were constructed using the neighbor-joining method. Red circles indicate the four complete PEDV genomes in this study.

substitution (535L→I) in the neutralizing epitope and was first discovered herein. Whether the above mentioned mutations influence the antigenicity and pathogenicity of novel PEDVs from Tibetan piglets awaits investigation.

Until now, the four different subtypes, G1a, G1b, G2a and G2b, have been identified in PEDV based on the S gene, each of which possessed unique epidemic features in specific regions and periods (Wen et al., 2018). The emergence of the novel subtype in PEDV often made it more difficult in prevention and control of this disease, and promoted the development of vaccines (Chang et al., 2017). At present, PEDV G2a and G2b subtypes are the main circulating strains in China (Wen et al., 2018). In this study, the phylogenetic tree based on the S genes revealed that the two novel subtypes of PEDV were identified in Tibetan piglets. Vlasova et al. (2014a) reported the potential for increased or altered virulence in PEDV as a result of recombination in S gene. In S genes, recombination events usually occurred in S1 or between S1 and S2 (Chiou et al., 2017; Vlasova et al., 2014a). In this study, two S genes of PEDVs from Tibetan pig had the same cross-recombination event in the S2 region, whose putative parental strains belonging to G2 subtype were epidemic strains identified in China in 2012 and 2017. The remaining five S genes had the same recombinant breakpoint in S1 region, whose putative parental strains belonging to G1 and G2 subtypes were epidemic strains isolated from China from 2011 to 2017. This novel recombination event occurred in seven S genes contributed to the novel subtypes of PEDV emerged in Tibetan pigs. Recently, Tibetan pigs are frequently transported and traded between the Qinghai-Tibet Plateau and other regions in China, which resulted in the rapid transmission and accelerating mutation of PEDV in Tibetan pigs. Hence, the recombination events of S gene in PEDV were more diversity and complex in Tibetan pigs, which was obvious different to that of previously reports in China (Lin et al., 2016). The novel recombination event in S genes of PEDV from Tibetan pigs further contributed to our understanding of the evolution and genetic diversity of PEDV from the Tibetan pigs. Notably, the same subtype and recombination pattern of S genes emerged in different Tibetan pig farms in different regions, which suggested that the new subtypes of PEDV strains have spread and circulated in Tibetan pigs.

The complete genomic sequences of the CH/TP-2-2/2018, CH/TP-3-1/2018, CH/TP-4-3/2018 and CH/TP-4-4/2018 strains obtained in this study were used to fully explore the genetic variation and evolutionary relationships among the Tibetan pig PEDVs. Although the nucleotide and amino acid sequences of the four complete PEDV genomes from the Tibetan piglets shared similarly high identities scores with the other reported PEDV strains, the four complete genomes formed a new cluster

in the phylogenetic tree, further indicating that the Tibetan pig PEDV strains were a novel strain. We also found that the phylogenetic relationships of ORF1a, E and M genes were similar to those seen in the tree based on the entire genome sequences of the Tibetan pig PEDVs, which all belonged to the G2b cluster. In contrast, the phylogenetic trees for the S, ORF1b, ORF3 and N genes displayed different genetic evolutionary relationships unlike that for the whole-genome, and the four genes belonged to the G2a cluster. In previous reports, the phylogenetic relationships of each gene in six U.S. PEDV strains were consistent with the complete genome (Chen et al., 2014b). Moreover, only the N gene or S gene from several Chinese strains showed a different evolutionary relationship in their phylogenetic trees compared with the full-length genome and other genes (Fan et al., 2017; Yang et al., 2013b). The differences we noticed in the phylogenetic relationships of all the genes from the PEDV strains were significant compared with those of these reported PEDV variants. Hence, we supposed that the more recombination phenomenon events might occurred in genomic sequence of PEDV from Tibetan pigs.

Genetic recombination introduces heterogeneity into the genome and plays a critical role in virus evolution (Wang et al., 2016). Here, we found that CH/TP-2-2/2018, CH/TP-3-1/2018, CH/TP-4-3/2018 and CH/TP-4-4/2018 strains were probably recombinants from those located on the G2a and G2b subgroups. This result is similar to previous findings demonstrating that recombination is a common phenomenon among PEDV variants (Lin et al., 2016). However, an obvious difference was identified in the recombination events in four PEDV genomes. The four recombinants had an identical crossover regional recombination event spanning S2, ORF3 and E genes. This is the first report of the crossover regional recombination event in PEDV genome, which may be an important contributor to the genetic diversity of PEDV in the Tibetan pigs. In recent years, for breeding improvements, Tibetan pigs have often been used for artificial insemination with Duroc or Landrace pigs. We speculate that these factors may lead to the occurrence, prevalence and complex recombination phenomenon we have seen with PEDV. Thus, it is necessary to monitor PEDV infected Tibetan pig herd for controlling the spread of the new subtypes PEDV.

5. Conclusions

We found that PEDV is still a major diarrhea-related pathogen in Tibetan pigs in this study. The novel PEDVs were discovered and circulating in Tibetan pigs on the Qinghai-Tibet Plateau. Meanwhile, the recombination events occurring in S genes were original from G1 and G2 subtypes PEDV epidemic strains from the Tibetan pig. Also, a crossover regional recombination event spanning S2, ORF3 and E genes was first detected in the four genomic sequences of PEDV from the Tibetan pigs. Therefore, these data provided further information on the prevalence and molecular characteristics of the novel PEDV in Tibetan pigs, contributing to future studies on the pathogenesis of PEDV in Tibetan pigs.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197652.

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